

# Immunoexpression of vascular endothelial growth factor and Ki-67 in human gingival samples: An observational study

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## Abstract

**Aim:** To evaluate immunohistochemically vascular endothelial growth factor (VEGF) and Ki-67 in human gingival samples and to compare these factors between healthy and diabetic patients. **Materials and Methods:** A total of 50 subjects were included in the study. They were categorized into three groups: Periodontally healthy group, periodontally diseased gingiva without any systemic disease group and periodontally diseased gingiva with controlled type II diabetes mellitus (DM) group. Gingival biopsies were performed and immunohistochemical analysis were done for VEGF and Ki-67 staining in gingival samples. **Results:** The present study found moderate intensity staining for VEGF in periodontitis group and periodontitis with controlled type II DM group and mild intensity staining for VEGF in periodontally healthy group. With regard to Ki-67, negative staining was observed in periodontally healthy group and mild staining in periodontitis group and periodontitis with controlled type II DM group. **Conclusion:** Further investigation needs to be conducted to identify how VEGF and Ki-67 are involved in the tissue inflammation associated processes and the relationship between VEGF and Ki-67 in progression of periodontitis.

**Key words:** Angiogenesis, diabetes mellitus, vascular endothelial growth factor

## INTRODUCTION

Angiogenesis is the formation of new capillaries by the budding of endothelial cells resident in the surrounding preexisting vessels. It is a complex process that involves endothelial cell division, selective degradation of vascular basement membranes and of surrounding extracellular matrix and endothelial cell migration. Periodontitis is an infection of highly vascularised supporting tissues of the teeth characterized by active and quiescent periods. Periodontitis is the sixth complication of diabetes mellitus (DM). DM contribute to periodontitis by mechanisms such as vascular changes, neutrophil dysfunction, altered collagen synthesis and genetic predisposition. Vascular endothelial growth factor (VEGF) appear to play an important role in the mediating diabetic vasculopathy in many organs. It also affects glucose levels resulting

in diabetic complications.<sup>[1]</sup> It has been a primary initiator and potential mediator of proliferative and nonproliferative diabetic retinopathy, neuropathy and nephropathy.

Vascular endothelial growth factor interacts with humoral factors that regulate bone homeostasis and bone development such as the recruitment of osteoblasts and osteoclasts. Bone formation and regeneration process are also linked to angiogenesis, a process in which VEGF is a major stimulant. Nevertheless, in periodontal tissues, angiogenesis seems to be important for the maintenance of tissue health and in periodontal diseases, VEGF is an important factor in the initiation and progression of gingivitis to periodontitis, promoting the expansion of the vascular network. Abberant angiogenesis is often associated with lesion formation in chronic periodontitis.

The periodontal vasculature is profoundly affected during progression of periodontal disease. Early

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in the progression, the perivascular connective tissue become disrupted, the collagenous fibers are destroyed, creating spaces within the tissue which are quickly filled by inflammatory cells and loose connective tissue. Capillaries then proliferate into spaces created in the loose connective tissue by angiogenesis. Angiogenesis also contribute to severity of inflammation. VEGF expression may be induced by several inflammatory mediators including prostaglandin E<sub>2</sub>, interleukin 6 and interleukin 1. In addition VEGF expression is reported to be regulated by the oxygen concentration of tissues, with hypoxia inducing its expression.<sup>[2]</sup>

Ki-67 is very important in the inflammation process. The Ki-67 antigen specific antibody is used in probes to detect cells undergoing division, which is a typical part of the inflammation. Ki-67 antigen expression has been detected in the nuclei of proliferation cells in the G<sub>1</sub>, S, G<sub>2</sub> and M phases of the cell cycle, but it is absent in inactive cells.<sup>[3]</sup>

The aim of the study was to detect the presence of VEGF and Ki-67 in gingival biopsy of periodontally healthy patients, periodontitis patients without any systemic disease and periodontitis patients with controlled type II DM.

## MATERIALS AND METHODS

The study included 50 patients who reported to the outpatient Department of Periodontics, M S Ramaiah Dental College and Hospital, Bangalore, Karnataka, with age ranging between 18 and 65 years. A total of 50 gingival samples were obtained. They were divided into three groups. Group 1 included periodontally healthy patients (10 gingival samples) with no sites with pocket depth and clinical attachment levels >3 mm and they exhibited <20% of sites with gingival bleeding and bleeding on probing. Group 2 included patients with periodontally diseased gingiva without any systemic disease (20 gingival samples) and Group 3 included patients with periodontally diseased gingiva with controlled type II DM (20 gingival samples). In Group 2 and Group 3 patients were with a minimum of six teeth with at least one site each with probing depth and clinical attachment loss >5 mm, >30% of sites with probing depth and clinical attachment loss >4 mm and presence of bleeding on probing. While subjects who were pregnant and lactating, on antibiotic therapy, smokers, on long term administration of anti-inflammatory medication, on localized radiation therapy of the oral cavity and on antineoplastic chemotherapy were excluded.

Gingival biopsies were performed with number 15 BP blades around the teeth and the gingival biopsy dimensions were 1.5 mm thick and 2–3 mm high. In patients with periodontitis without any systemic disease and periodontitis with controlled type II DM, gingival biopsies were performed during periodontal surgery after completion of phase I therapy (scaling and root planning). In periodontally healthy patients, gingival biopsies were taken during crown lengthening, operculectomy and esthetic surgical procedures. After retrieval biopsies were immediately fixed in 10% buffered formalin, processed and stored as paraffin blocks.

## Immunohistochemistry

From each biopsy, two sections about 3 µm thick sections were prepared and mounted on silane coated slides. One section was used for VEGF staining and the other for Ki-67 staining. Paraffin sections were dewaxed using xylene, rehydrated and washed in phosphate buffered saline (pH 7.4) for 10 min. To unmask the antigens a microwave oven and a 2.1% citric acid solution were used with antibodies VEGF and Ki-67. Sections were incubated with primary antibody for 30 min at room temperature. Slides were rinsed in buffer, and immunoreactions were completed with the anti-mouse/rabbit IgG – Poly horse radish peroxidase method using a kit (Novocrasta UK) and a multilink as a secondary biotinylated antibody. After incubation with D-amino benzidine chromogen, the specimens were counterstained with hematoxylin and coverslipped. A positive control was used. A negative control using the secondary antibody without the primary one is used. All stained slides were examined at ×40 and ×10 and positivity was determined. Immunostaining of VEGF was localized in the cytoplasm of macrophages and fibroblasts. Ki-67 staining was noticed in the nucleus. The intensity of positivity was estimated as:<sup>[4]</sup> Negative (–): Fewer than 5% positive cells or no staining; Grade (+): Mild - 5–24% positive cells; Grade (++): Moderate - 25% to 50% positive cells and Grade (+++): Severe - >50% positive.

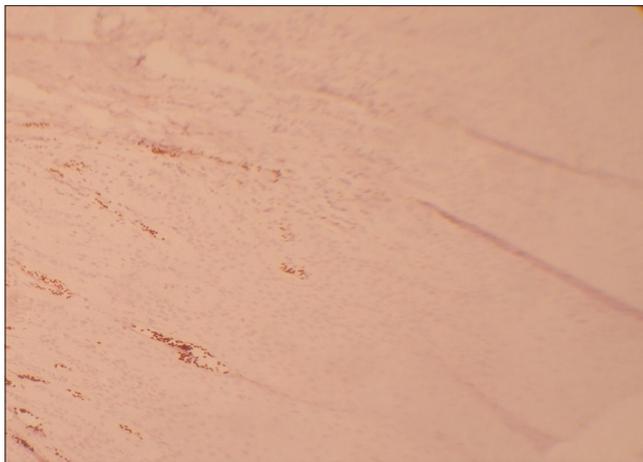
Proportions were compared using Chi-square test of significance. One-way analyses of variance was used to test the difference between groups. The Student's *t*-test was used to determine whether there was a statistical difference between study groups in the parameters measured. Data analysis was carried out using Statistical Package for Social Science (SPSS, version 10.5, IBM Chicago, IL, USA) package.

## RESULTS

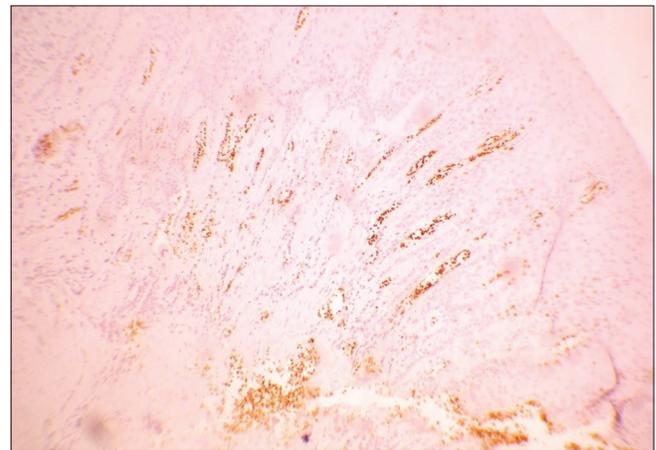
The intensity of VEGF staining in 10 patients with periodontally healthy gingival showed mild

staining (Grade +) [Figure 1]. In patients with periodontally diseased gingiva without any systemic disease, 20% of gingival biopsies showed mild staining (Grade +) and 80% of them showed moderate staining (Grade ++). In patients with periodontally diseased gingiva with controlled type II DM, 100% of gingival biopsies showed moderate staining (Grade ++). In 50 gingival biopsy samples, 72% of them showed moderate staining (Grade ++). In patients with periodontally diseased gingiva without any systemic disease, 20% of gingival biopsies showed mild staining (Grade +) and 80% of them showed moderate staining (Grade ++). In patients with periodontally diseased gingiva with controlled type II DM, all the gingival biopsies showed moderate staining (Grade ++). The *P* value obtained was < 0.001 and the intensity of VEGF staining between three groups were found to be statistically significant.

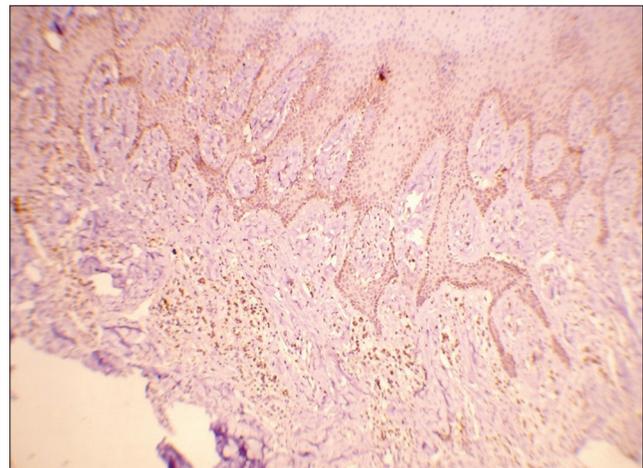
In patients with periodontally diseased gingiva without any systemic disease, 10% of gingival biopsies stained negative and 90% of them showed mild staining (Grade +). In patients with periodontally diseased gingiva with controlled type II DM, 100% of gingival biopsies showed mild staining (Grade +). In a total of 50 gingival biopsy samples, 24% of them showed negative staining for Ki-67 and 76% of them showed mild staining (Grade +). *P* value obtained was < 0.001 and the intensity of Ki-67 staining between three groups were found to be statistically significant. The intensity of Ki-67 staining in patients with periodontally diseased gingiva without any systemic disease showed negative staining



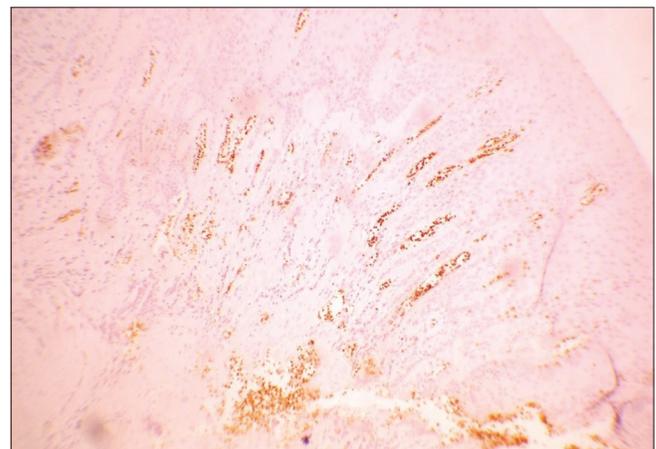
**Figure 1:** Vascular endothelial growth factor - Mild (Grade +) staining in Group 1



**Figure 2:** Vascular endothelial growth factor - Moderate (Grade ++) staining in Group 2



**Figure 3:** Vascular endothelial growth factor - Moderate (Grade ++) staining in Group 3



**Figure 4:** Ki-67 - Negative staining in Group 1

in 10% of gingival biopsies and 90% of gingival biopsies showed mild staining (Grade +). In patients with periodontally diseased gingiva with controlled type II DM, all the gingival biopsies showed mild staining (Grade +) for Ki-67. The *P* value obtained was 0.147 and the intensity of staining was not found to be statistically significant on comparison of Ki-67 staining between periodontitis group and periodontitis with controlled type II DM group [Graph 2].

## DISCUSSION

Angiogenesis is regulated through a complex interplay of molecular signals mediated by growth factors involving extracellular matrix remodelling, endothelial cell migration and proliferation, capillary differentiation and anastomosis. As demonstrated in this study, immunostaining of VEGF is localized in the cytoplasm of macrophages and fibroblasts and this is consistent with previous studies by Fukumura *et al.*, which demonstrated that macrophages and

fibroblasts were the major source of synthesis and secretion of VEGF.<sup>[5]</sup> Unlü *et al.*, conducted a study and observed no expression of VEGF in negative healthy controls (patients without any systemic or periodontal diseases or in the healthy periodontal tissues of periodontal patients without DM). However VEGF was detected in diseased periodontal tissues both in diabetic and nondiabetic patients, and in healthy tissues of diabetic patients.<sup>[6]</sup>

Booth *et al.*, have suggested that VEGF was generally unregulated even in relatively healthy sites, probably either reflecting subclinical levels of inflammation/healing after the microbial assault, or revealing the presence of VEGF as a component of physiological angiogenesis in the gingival/periodontal environment.<sup>[7]</sup> In our study, we observed positive VEGF staining mostly in monocytes, macrophages and to a lesser degree in endothelial cells. This observation was in concordance with previous reports by Nakagwa that have reported the presence of VEGF within these cells.<sup>[8]</sup> Walttenberger has shown the impairment of monocytic migration towards a gradient of VEGF in diabetics, whereas our results indicated the contrary; presence of VEGF staining in the monocytes in DM.<sup>[9]</sup>

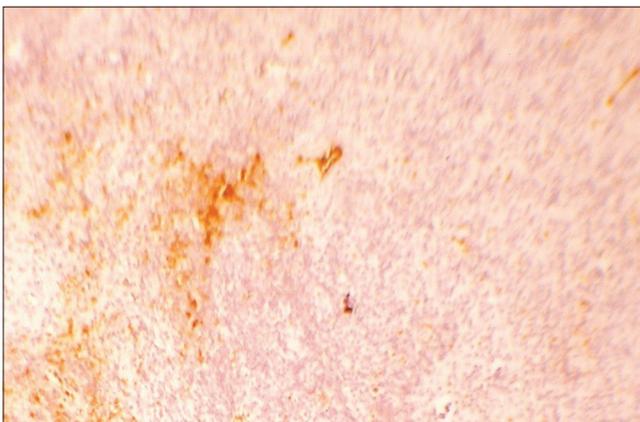


Figure 5: Ki-67 - Mild (Grade +) staining in Group 2

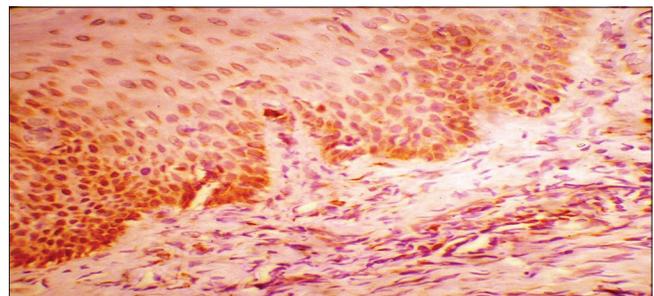
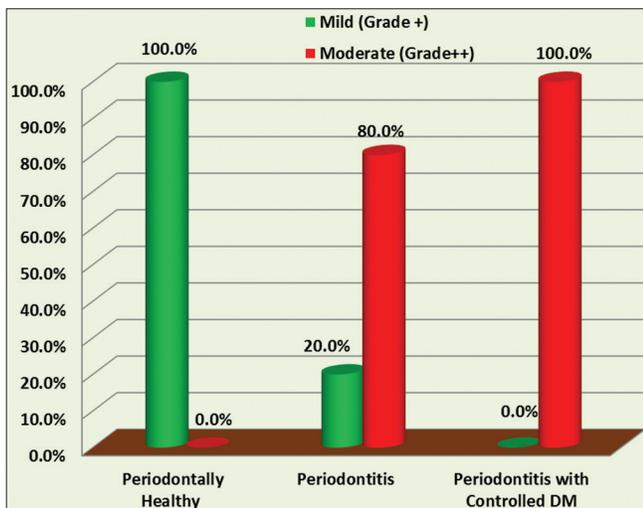
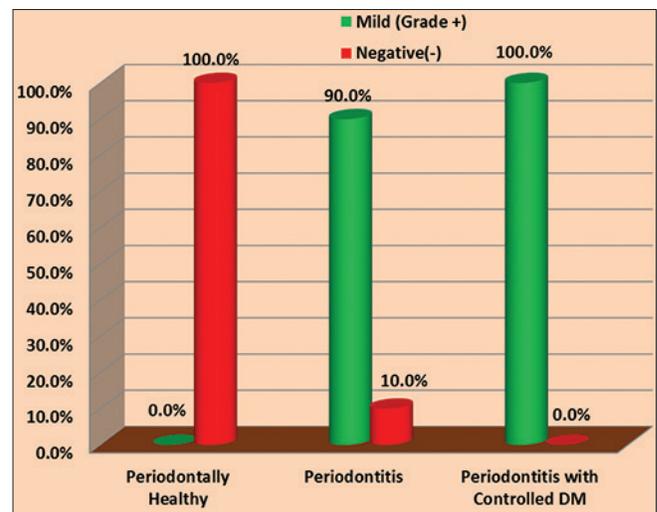


Figure 6: Ki-67 - Mild staining (Grade +) in Group 3



Graph 1: Intensity of VEGF staining in study groups



Graph 2: Intensity of Ki-67 staining in study groups

Diabetes mellitus and periodontitis represent common chronic diseases that may have a reciprocal influence. It has been demonstrated that DM may have an inductive effect on the VEGF levels of the periodontium during periodontal disease. Therefore, VEGF is related to important diabetic microvascular complications such as tissue ischemia, angiogenesis, permeability in many organs, and alterations in blood glucose levels. An increased epithelial VEGF expression in patients with type I DM and type II DM compared to controls was observed in study done by Asprielloo *et al.* and this result further confirmed that DM has an inductive effect on periodontal VEGF. In patients with diabetes, VEGF overexpression plays a primary role in promoting the extravasation of inflammatory cells, suggesting a useful antiangiogenic strategy for periodontitis treatment.<sup>[10]</sup> Periodontitis may exacerbate diabetes by decreasing glycemic control, indicating a degree of synergism between the two diseases.<sup>[11]</sup> This was also observed in our study, revealing higher presence of VEGF in diabetics with periodontal disease compared to healthy gingival tissues from nondiabetic periodontal patients.

In the present study moderate (Grade II) expression of VEGF was seen in gingival samples of patients with controlled type II DM and patients with

periodontitis than periodontally healthy patients. In this study none of the groups showed severe (Grade III) VEGF expression. It may be because gingival biopsies were taken after scaling and root planning which has led to the reduction in intensity of inflammation. A study done by Unlü *et al.* showed that VEGF is increased in gingival tissues of diabetic patients, especially those with periodontitis.<sup>[6]</sup>

Ki-67 is a nuclear protein which is expressed in proliferating cells. It can be used as a marker to estimate tissue growth. A study done by Sagun compared gingival biopsies from hereditary gingival fibromatosis patients and healthy gingival biopsies and found that the number of Ki-67 antigen positive cells nuclei was observed to be low in the basal cell layers of hyperplastic epithelia and it was similar to the observation seen in gingival samples from healthy control group.<sup>[12]</sup>

Nagarakanti *et al.*, found a significant increase in the proliferative marker (Ki-67) in periodontitis tissue samples versus healthy gingiva. They reported that the oral gingival epithelium should be the main source of this proliferation and differentiation. Major cells that stained for Ki-67 were fibroblast-like. Very few T and B-cells were positive in periodontal lesions. Ki-67 is regarded as a good marker to estimate the

state of tissue growth.<sup>[13]</sup> Saito *et al.*, found that mean rates of Ki-67 positive cells in the nifedipine and phenytoin were significantly higher than in healthy tissues.<sup>[14]</sup> The continuous renewing of gingival tissues, the kinetics, cytodifferentiation and the importance of keratinisation increases especially in diabetic patients. Enhanced inflammatory response leads to inadequate keratinisation and results in severe periodontal disease. Açıkgoz *et al.*<sup>[15]</sup> found no significant difference in manner of keratinocyte proliferation between diabetic and healthy group. He also concluded that type II diabetes is not associated with the severe disease of gingival tissue and the diabetes does not have an additional effect on the mitotic activity of gingival keratinocytes. In this study intensity of Ki-67 staining was found to be negative in gingival biopsy samples from periodontally healthy patients and mild staining (Grade +) was noticed in periodontitis and periodontitis with type II DM.

## CONCLUSION

The conclusion drawn from this study are the intensity of VEGF staining between three groups was found to be statistically significant. The intensity of VEGF staining was found to be mild (Grade +) in Group 1 and moderate (Grade ++) in Group 2 and Group 3 and the intensity of VEGF staining was found to be statistically significant on comparison of VEGF staining between periodontitis Group and periodontitis with controlled type II DM Group. The intensity of Ki-67 staining between three groups was also found to be statistically significant. The intensity of Ki-67 staining in Group 1 was found to be negative but in Group 2 and Group 3, Ki-67 staining was found to be mild (Grade +) and the intensity of Ki-67 staining was not found to be statistically significant on comparison of Ki-67 staining between periodontitis Group and periodontitis with controlled type II DM Group. VEGF is an important factor in the pathogenesis of periodontitis. When angiogenesis is stimulated and the vascular network grows, this leads to edema and results in decrease in blood flow. Ki-67 is a well recognised nuclear proliferation marker. There seems to be an increase in Ki-67 with increase in tissue proliferation. Investigations in large population need to be conducted to identify how VEGF and Ki-67 are involved in the tissue inflammation associated processes and the relationship between VEGF and Ki-67 in progression of periodontitis.

## CLINICAL IMPLICATION

Vascular endothelial growth factor acts as a potent and pleiotropic inflammatory agent in periodontitis,

especially when further aggravated by diabetes, suggesting a useful anti angiogenic strategy for periodontitis treatment. A key area of ongoing research will be the role of VEGF in gingival disease. It is likely that new insights into the importance of VEGFs for disease will continue to be generated. Consequently, the scope for using anti-VEGF approaches therapeutically will grow, and the challenge will be to develop more effective and economic ways to prevent VEGF-driven pathophysiological angiogenesis.

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